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Low responsiveness of hepatitis B virus-transgenic mice in antibody response to T-cell-dependent antigen: defect in antigen-presenting activity of dendritic cells

S. M. F. AKBAR, M. ONJI, K. INABA,* K.-I. YAMAMURA† & Y. OHTA *Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, *Department of Zoology, Faculty of Science, Kyoto University, Kyoto and †Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto, Japan*

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SUMMARY

The experiments presented here were performed to evaluate immune responsiveness of hepatitis B virus (HBV)-transgenic mice (transgenic mice), as a model of HBV-carrier state to a T-cell-dependent antigen, keyhole limpet haemocyanin (KLH). The transgenic mice which were completely unresponsive to hepatitis B surface antigen (HBsAg), responded poorly to KLH. The levels of anti-KLH antibodies (Ab) produced *in vivo* were significantly lower in transgenic mice compared with the normal control mice at respective immunizing doses of KLH. In addition, a little or no anti-KLH Ab production was detected in culture supernatants of KLH-primed transgenic mice spleen cells. KLH-primed T cells from normal and transgenic mice induced anti-KLH Ab production from transgenic B cells in the presence of antigen-presenting spleen adherent cells (SAC) from normal mice, but not those from transgenic mice. Depletion of dendritic cells from normal mice-derived SAC completely abrogated the anti-KLH Ab response in transgenic spleen cell culture and their addition to the culture restored the response. Low efficiency of transgenic dendritic cells was demonstrated in sodium periodate ($NaIO_4$)-induced non-specific and allogenic antigen-induced T-cell proliferation. Finally, cytofluorometric analyses showed a reduced Ia antigen expression on transgenic dendritic cells. These results indicate that low responsiveness of transgenic mice in specific-antibody response is not due to functional defects in T cells or B cells but rather to a defect of antigen-presenting activity of dendritic cells.

INTRODUCTION

Both cellular and humoral immune responses to hepatitis B virus (HBV)-related antigens have been reported to be impaired in human HBV carriers.[1–5] Earlier studies *in vitro* have documented abnormalities in functions of helper or regulatory T cells[2] and the inability of B cells to produce specific-antibody (Ab)[2,4,5] in response to hepatitis B surface antigen (HBsAg). As a result of recent progress in gene technology, transgenic animals bearing HBV genes as a model of HBV carrier state have become available[6] and experiments with HBV-transgenic mice (transgenic mice) have revealed that the low responsiveness of transgenic mice in specific-Ab production to HBV-related antigen is due to tolerance at the T-cell level.[7]

However, immunosuppressive serum factor(s) detected in patients with acute HBV infection before the onset of hepatitis have been reported to inhibit the functions of T and B lymphocytes in normal individuals.[8] Marked impairment of polyclonal IgG secretion and T-cell function are also shown not only in HBV carriers with hepatitis[5] but also in asymptomatic HBV carriers.[9] Thus, it is not still clear whether the immunlogical abnormalities in HBV carriers are due to tolerance, factors related to HBV or secondary to hepatitis.

On the other hand, studies have demonstrated that infection with HBV and/or its expression products may interfere with the immune response by infecting mononuclear cells,[10] modulating interferon systems[11] and inhibiting the release of cytokines.[12] Furthermore, HBV-containing sera or purified Dane particles have been reported to suppress erythropoiesis[13] and inhibit the growth of haemapoietic cell line *in vitro*.[14]

This circumstantial evidence suggests that there may be inefficiency in the immune responsiveness of transgenic mice other than the attribution of specific T-cell tolerance.

Abbreviations: Ab, antibody; AMLR, allogenic mixed leucocyte reaction; anti-HBs, antibody to HBsAg; APC, antigen-presenting cell; C, complement; CFA, complete Freund's adjuvant; Con A, concanavalin A; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; Ig, immunoglobulin; i.p., intraperitoneal; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharide; mAb, monoclonal antibody; NaIO, sodium periodate; OD, optical density; PBS, phosphate-buffered saline; SAC, spleen adherent cell; SD, standard deviation; transgenic mice, HBV-transgenic mice.

Correspondence: Dr M. Onji, Third Dept. of Internal Medicine, Ehime University School of Medicine, Shigenobu, Ehime, 791-02 Japan.

Therefore, we attempted to re-evaluate responsiveness of transgenic mice to a HBV-unrelated, T-dependent antigen, keyhole limpet haemocyanin (KLH) by measuring specific-Ab production both *in vivo* and *in vitro*. We found that transgenic mice responded poorly *in vivo* to soluble KLH and produced significantly lower levels of anti-KLH Ab compared with normal control mice. In addition, spleen cells from KLH-primed transgenic mice hardly responded to secrete anti-KLH Ab *in vitro*.

However, this inefficiency of the immune response was not mainly due to a defect in functions of T cells and B cells. Based on these observations, *in vitro* experiments were undertaken to focus on the function of the antigen-presenting cell activity of spleen adherent cells (SAC) which included dendritic cells and macrophages. We found that a defect in antigen-presenting activity of transgenic dendritic cells was a major mechanism of inefficiency in the antigen-specific Ab response in transgenic mice.

MATERIALS AND METHODS

Animals

Transgenic mice (1.2HB-BS10) were produced by microinjecting a partial tandem duplication of the complete HBV genome into fertilized eggs of C57BL/6 mice.[6] These transgenic mice produced HBsAg and HBeAg, and viral genome was detected in the serum. Male transgenic and normal C57BL/6 mice (Charles River Inc, Nagoya, Japan) were used for immunization at 6–8 weeks old. Transgenic mice were housed separately from normal mice and both strains of mice were bred in our animal facility. C3H/He mice (H-2^k; Clea Japan Inc, Tokyo, Japan) were used as a donor of T cells in the allogenic mixed leucocyte reaction (AMLR).

Reagents

The antigens tested were KLH (Polyscience Inc., Warrington, PA) and HBsAg (Shionogi, Co., Osaka, Japan). Collagenase was purchased from Sigma (St Louis, MO). Concanavalin A (Con A) and lipopolysaccharide (LPS) (Sigma) were used for activation of T and B cells, respectively. Cytotoxicity medium (RPMI-1640 supplemented with 0·3% bovine plasma albumin and 25 mM HEPES) and lympholyte-M containing Ficoll 400 and sodium diatrizoate, density $1{\cdot}0875 \pm 0{\cdot}0005$ were obtained from Cedarlane Lab. Ltd (Hornby, Ontario, Canada).

Antibodies

Monoclonal antibodies (mAb) to Thy-1.2 (clone 5a-8), Lyt-1.2 (clone CG16) (Cedarlane), 33D1 [TIB227 from American Type Culture Collection (ATCC), Rockville, MD], J11d (TIB183; ATCC) and CD45R (clone RA3-3A1/6.1. TIB146; ATCC), Ia antigen (clone B17-123.R2; (Cedarlane) were used with low-tox rabbit complement (C) (Cedarlane) to deplete T cells, dendritic cells, B cells and Ia^+ cells, respectively. Anti-Ia monoclonal antibody (clone K25-87) (Cedarlane), anti-mouse CD11c (N418), a dendritic cell specific antibody,[15] fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Cedarlane), and FITC-conjugated anti-hamster IgG (Cappel, Malvern, PA) were used for staining cells. Goat $F(ab')_2$ fragment anti-mouse IgM and intact anti-mouse IgG (Fc) from Cappel and peroxidase-conjugated affinity-purified goat anti-mouse IgM and IgG from E.Y. Laboratories (San Mateo, CA) were used in enzyme-linked immunosorbent assay (ELISA).

Immunization

Mice were immunized subcutaneously with the graded doses of HBsAg (1–50 μg) in complete Freund's adjuvant (CFA) and boosted with the same doses of antigen 4 weeks later. For immunization with KLH, graded doses of KLH in phosphate-buffered saline (PBS) were intraperitoneally (i.p.) inoculated after a 2-week interval. Mice were bled before and at various time-points after primary and secondary immunization. In most of the experiments, mice were immunized with 5 μg of KLH i.p. and boosted once 2 weeks later.

Cell preparation

Various cell populations were prepared from mouse spleen according to methods described previously[16] with some modifications. Briefly, lymphocytes consisting of T and B cells (T/B cells) were obtained by passing spleen cells through Sephadex G-10 columns. In some experiments, T/B cells were treated with 33D1 + C to deplete dendritic cells. B cells were isolated from T/B cells by depleting T cells with anti-Thy-1.2 and anti-Lyt-1.2 + C. T cells were enriched by applying T/B cells to a T-cell recovery kit (Biotex Lab. Inc, Alberta, Canada). SAC consisting of dendritic cells and macrophages were prepared by incubating collagenase-digested spleen low-density cells for 2 hr as described previously.[17] To adjust cell number, adherent cells were recovered by incubation with 10 mM ethylinediamine tetraacetic acid (EDTA) in PBS and then treated with a mixture of anti-Thy-1.2, anti-Lyt-1.2 and anti-CD45R plus C to eliminate contaminating T and B cells, respectively. In this population, 45–60% of cells were found to be macrophages by phagocytosis of latex particles.

Enriched poulations of dendritic cells were recovered as non-adherent cells after overnight culture of SAC and they were depleted of Fc receptor-bearing cells by rosette formation with Ab-coated sheep erythrocytes. The remaining adherent cells after overnight culture of SAC were macrophages. They were dislodged by treatment with 10 mM EDTA in PBS for 20 min These three types of cell population were treated with 40 μg/ml of mitomycin C for 30 min prior to use as antigen-presenting cells (APC).

Cell culture for antibody production and proliferation

For antibody production *in vitro*, 5×10^6 spleen cells from KLH-primed mice were cultured in 1 ml of RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 50 μM 2-mercaptoethanol (2-ME) and 20 μg/ml gentamycin sulphate in the presence or absence of KLH at 0·5 μg/ml in 24-well culture plates for 10 days. In some experiments, T/B cells (5×10^6) or a mixture of T cells (2×10^6) and B cells (3×10^6) in combination with APC (5×10^4–10^5) were used instead of bulk spleen cells. At the end of culture, the supernatants were collected from each well, centrifuged and filtered. One-half aliquots of all samples were stored at 4° until assay, usually within 2–3 days, and the other halves were stored at −20° to assay once again after the end of the whole experiment. For non-specific immunoglobulin

(Ig) production by B cells, purified B cells at 3×10^5 cells/well were cultured with LPS (30 μg/ml) in 200 μl of medium in 96-well culture plates, and culture supernatants were collected 5 days later.

Proliferation of T cells in response to Con A (2 μg/ml) and that of B cells to LPS (50 μg/ml) were assayed by measuring [^{3}H]thymidine incorporation (1 μCi/ml) during the last 12 hr of culture period of 56 and 72 hr, respectively.

Stimulatory activity of various doses of dendritic cells was also determined by monitoring proliferation of KLH-primed T cells, $NaIO_4$-treated normal C57BL/6 T cells[18] and allogenic (C3H/He) T cells. DNA synthesis was assayed by [^{3}H]thymidine incorporation (1 μCi/ml) for 14 hr before the end of culture period. All of the proliferation assays were performed in 96-well flat-bottomed plates.

Detection of antibody response

KLH-specific and LPS-induced non-specific IgM and IgG productions were determined by ELISA according to the standard indirect procedure and antibody sandwich method, respectively, with some modifications.[19] Binding capacities of peroxidase-conjugated goat anti-mouse IgM and IgG were assessed as enzymatic reactions of hydrolysis of a substrate, orthopheylenediamine, by measuring the optical density (OD) value at 492 nm with an ELISA reader (SJeia Auto Reader, Model ER-8000, Sanko Junyaku Co., Ltd, Tokyo, Japan). Pooled sera from unprimed mice were used as a negative control, and sera containing a known amount of IgM or IgG Ab (Binding Site Limited, Birmingham, U.K.) and standard sera rich in either anti-KLH IgM or anti-KLH IgG Ab were used as positive controls. In KLH-specific Ab assay, the cut-off value for a positive response was the mean ± 3 SD of the OD value for the negative control. Since the OD values at 1:32,000 and 1:128,000 dilutions of anti-KLH IgM-rich and IgG-rich standard sera were almost equal to the respective cut-off values, their OD values were designated as 1 DTm U and 1 DTg U, respectively. Levels of anti-KLH Ab were estimated based on the standard curve plotting OD values at different dilutions of anti-KLH Ab-rich standard serum.

Antibody to HBsAg (anti-HBs) was also measured by ELISA using an anti-HBs kit (Shionogi), in which developing reagent was peroxidase-labelled HBsAg and substrate was tetramethyl benzidine dihydrochloride. OD values were measured at 655 nm, and the mean OD for negative controls plus 0·040 was taken as the cut-off value based on the instruction. Results were expressed as cut-off index, calculated by dividing OD values of the samples by the cut-off value. A value of 1·0 or more of cut-off index was considered as a positive anti-HBs response.

Cytofluorometry

This was done according to a previously described procedure with slight modifications.[20] Briefly cells were stained with optimum dilutions of primary antibody, followed by FITC-conjugated antibody, washed and suspended in PBS containing 1% bovine serum albumin (BSA) and 0·02% sodium azide and finally FITC$^+$ cells were counted in a flow cytometer (Epics profile, Coulter Corp. Hialeah, FL). Cells unstained and stained with FITC-conjugated second Ab alone served as controls.

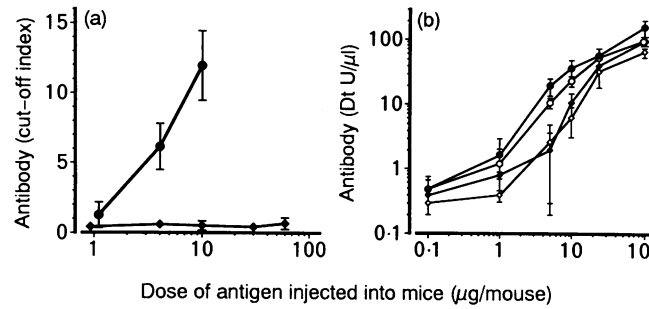

Figure 1. Low responsiveness of HBV-transgenic mice to the stimulation with KLH that were unresponsive to immunization with HBsAg. Production of total anti-HBs Ab [closed symbols (a)], anti-KLM IgM [open symbols (b)] and anti-KLH IgG [closed symbols (b)] in sera were determined in normal C57BL/6 mice (circles) and HBV-transgenic mice (diamonds) 2 weeks after secondary immunization with graded doses of HBsAg emulsified in CFA and soluble KLH in PBS. The levels of antibody were shown as cut-off index (a) and DT U/μl (b), and values of 1·0 or more were considered as positive. Each group consisted of 10 mice. Symbols and bars represent the mean ± SD of anti-HBS (a) and anti-KLH Ab (b) produced in each group, respectively. In respective immunizing doses of KLH, HBV-transgenic mice produced significantly lower levels of both anti-KLH IgM and IgG Ab compared with normal mice ($P < 0{\cdot}05$, Student's *t*-test).

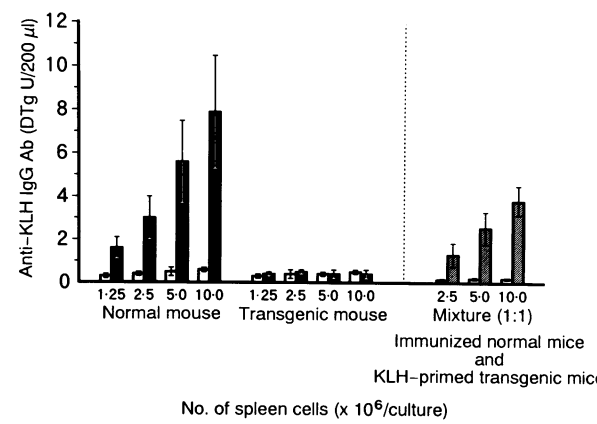

Figure 2. Comparison of anti-KLH IgG Ab response *in vitro* between KLH-primed normal mice and HBV-transgenic mice. Normal and HBV-transgenic mice, five of each were immunized i.p. twice with 5 μg of KLH in PBS in 2-week intervals and killed 8 months after secondary immunization. Spleen cell suspensions from individual mice were made and various numbers of them were cultured separately in the presence (■) or absence (□) of 0·5 μg/ml of KLH in 1 ml of culture medium for 10 days. The mixture (1:1) indicates the culture containing equal numbers of spleen cells from KLH-primed transgenic mice and unimmunized normal mice. The number of cells indicates the total number of cells in the culture. The mixture was cultured at different cell numbers with (■) or without (□) KLH for 10 days. Anti-KLH IgG Ab in culture supernatant was measured by ELISA. Values were expressed as the mean DTg U/200 μl of culture supernatants of duplicate assays of triplicate cultures and bars represent the SD of the mean. Values of 1·0 or more were considered as positive.

RESULTS

Responsiveness of HBV-transgenic mice to KLH *in vivo*

With regard to specific-Ab production, transgenic mice were not only unresponsive to HBsAg but also responded poorly to

Table 1. Defect in antigen-presenting activity of spleen adherent cells from HBV-transgenic mice

Cells prepared from*					
Whole (5×10^6)	T/B cells (5×10^6)	B cells (3×10^6)	T cells (2×10^6)	SAC (10^5)	Anti-KLH IgG antibody (DTg U/200 μl)†
TM					0·4±0·1
TM				NM	3·2±1·0
	TM				0·5±0·3
	TM			TM	0·5±0·2
	TM			NM	3·3±0·7
		TM	TM	TM	0·6±0·2
		TM	TM	NM	3·2±0·8
		TM	NM	TM	0·7±0·2
		TM	NM	NM	4·4±1·7

* Normal (NM) and HBV-transgenic (TM) mice were immunized i.p. twice with 5 μg of KLH in PBS after a 2-week interval and used for the experiment 8 months after last injection. T/B cells, G10-non-adherent cells; B cells, G10-non-adherent cells depleted of Thy-1.2$^+$ and Lyt-1.2$^+$ cells; T cells, G10-non-adherent cells recovered from affinity column which specifically absorbed B cells; SAC, mixture of dendritic cells and macrophages obtained by detaching SAC with EDTA treatment. In this population, about 30–42% cells were found to be Ia$^+$ and 25–34% cells were dendritic cells as judged by indirect staining with anti-mouse C11c (N418).[15]

† Culture supernatant from each well was collected 10 days after the onset of culture of cells indicated in the presence of 0·5 μg/ml of KLH. Anti-KLH IgG antibody in the culture supernatants were determined by ELISA method. Levels of anti-KLH IgG were expressed as DTg U/200 μl of supernatant. Each value represents the mean ± SD of the duplicate assays of triplicate cultures of five experiments. Values 1·0 or more were considered as positive.

KLH, an HBV-unrelated antigen. The transgenic mice could not produce anti-HBs even when injected with high doses of HBsAg in CFA (Fig. 1a), compatible with the findings reported in HBV carriers using HBV-related antigens.[1,3,7] These transgenic mice produced anti-KLH Ab as the doses of KLH were increased; however, the levels of their responses were significantly lower compared with normal mice at respective doses of KLH [dose of KLH, anti-KLH Ab (mean ± SD of 10 mice) in DT U/μl, normal mice versus transgenic mice: anti-KLH IgM, 0·1 μg, 0·5±0·3 versus 0·3±0·1; 1·0 μg, 1·2±0·9 versus 0·4±0·1; 5·0 μg, 11·0±2·0 versus 2·7±2·4; 10 μg, 24·0±5·0 versus 6·2±2·7; 25 μg, 55·0±15·0 versus 34·0±15·0; 100 μg, 102·0±24·0 versus 68·0±23·0; anti-KLH IgG in DTg U/μl: 0·1 μg, 0·5±0·2 versus 0·34±0·1; 1·0 μg, 1·7±1·4 versus 0·8±0·1; 5·0 μg, 21·0±7·0 versus 2·0±1·8; 10 μg, 38·0±13·0 versus 11·0±5·0; 25 μg, 64·0±21·0 versus 43·0±11·0; 100 μg, 168·0±44·0 versus 101·0±26·0] (Fig. 1b) and HBV-transgenic mice required 3–5 times more KLH than normal mice to give comparable levels of anti-KLH Ab response. Reduced response appeared to be much in evidence when transgenic mice were immunized with low doses of KLH.

Little anti-KLH Ab production by KLH-primed transgenic mice spleen cells *in vitro*

In the next set of experiments, anti-KLH Ab production by KLH-primed spleen cells was examined *in vitro*. After injecting 5 μg of KLH twice at 2-week intervals, spleen cells were cultured in the presence or absence of 0·5 μg/ml of KLH for 10 days. When the cells were cultured from normal mice 2 weeks after the secondary immunization, anti-KLH IgG Ab was detected without exogenous KLH in culture (spontaneous) in all normal mice tested. In contrast, spleen cells from two of 10 transgenic mice produced anti-KLH IgG Ab, and the levels were much lower compared with normal mice. No anti-KLH Ab production was detected in the rest of transgenic mice even culturing in the presence of exogenous KLH (not shown). These results seem to demonstrate either that lymphocytes were not primed adequately with KLH in transgenic mice *in vivo* or that KLH-primed lymphcoytes were not reactivated sufficiently *in vitro*.

The kinetics of anti-KLH Ab production in normal mice revealed that immunizing normal mice with 5 μg of KLH was sufficient to have anti-KLH Ab production in all the normal mice tested both *in vivo* and *in vitro*. *In vitro*, spontaneous production (without the addition of exogenous KLH in culture) of KLH-specific Ab was observed in the culture of bulk population of spleen cells from normal mice until 4–5 months after the last immunization with 5 μg of KLH and was decreased thereafter, and no spontaneous production of anti-KLH Ab was seen 7–8 months after the last immunization. But spleen cells from KLH-primed normal mice produced anti-KLH Ab 7–8 months after the last immunization when an adequate amount of exogenous KLH was added to the culture. Therefore, spleen cells from mice that had been primed with 5 μg of KLH 7–8 months previously were used in the following experiments and shown to have a KLH-induced anti-KLH Ab response.

As shown in Fig. 2, KLH-primed normal mice produced anti-KLH IgG Ab in the presence of KLH during the culture and the level of anti-KLH IgG Ab was proportional to the number of spleen cells in culture. In contrast, spleen cells from KLH-primed transgenic mice could not produce anti-KLH IgG Ab even in the presence of exogenous KLH in culture. However, when the spleen cells from KLH-primed transgenic mice and unimmunized normal mice were mixed (1 : 1), and cultured, then anti-KLH IgG Ab was produced in the culture, suggesting that transgenic mice-derived B cells were able to produce anti-KLH IgG Ab in collaboration with spleen cells from unimmunized normal mice. Unimmunized normal mice spleen cells could not produce anti-KLH Ab under any experimental conditions. No significant inefficiency in B-cell functions of transgenic mice compared with those of normal mice was demonstrated in proliferative (normal verus transgenic B cells, 47,408±12,718 c.p.m. versus 37,383±8953 c.p.m.), non-specific IgM (3·0±1·3 μg/ml versus 2·8±1·1 μg/ml) and IgG (2·8±1·7 μ/ml) versus (2·3±1·1 μg/ml) (mean±SD of five experiments; $P>0·1$) productions in response to LPS.

Defect in antigen presentation by spleen adherent cells from HBV-transgenic mice

To dissect the cell type responsible for inefficient responsiveness in transgenic mice, we carried out experiments by mixing various populations in spleen cell suspension from KLH-primed normal and transgenic mice (Table 1). Transgenic bulk spleen cells by themselves could not produce anti-KLH IgG Ab, while they produced anti-KLH IgG Ab when cultured with SAC from normal mice. Similar results were obtained when T/B cells or T cells and B cells from KLH-primed transgenic mice were co-

Table 2. Critical role of dendritic cells in anti-KLH antibody response

(A) From normal mice

APC*	Anti-KLH IgG antibody (Dtg U/200 μl)¶ T/B cells prepared from:** Normal mice	HBV-transgenic mice
None	0·9±0·5	0·5±0·3
SAC† treated with C alone	5·7±1·0	3·6±0·6
SAC treated with J11d and 33D1+C	1·1±0·4	0·5±0·3
SAC treated with anti-Ia+C	1·0±0·4	0·4±0·3
Macrophages‡	1·3±0·9	0·4±0·4
Dendritic cells§	5·7±0·4	3·9±0·5

(B) Combination

SAC	Dendritic cells	Macrophages	Normal mice	HBV-transgenic mice
None			1·1±0·4	0·5±0·2
NM			5·7±0·8	3·5±0·4
NM (treated with 33D1+C)			1·9±0·8	0·6±0·4
	NM		6·4±0·8	3·9±0·6
TM			1·3±0·4	0·5±0·3
	TM		2·2±0·3	0·9±0·4
NM		NM	5·3±0·9	3·7±0·5
NM		TM	5·7±0·3	3·8±0·4
TN		NM	1·4±0·5	0·5±0·2

* APC obtained from normal unimmunized mice were used in (A) and those from normal and transgenic mice were used in (B). NM, normal mice; TM, transgenic mice; SAC, spleen adherent cells.

† SAC were prepared by 2-hr adherence of $2·5 \times 10^6$ collagenase-digested, low-density spleen cells on to the surface of 24-well culture plates. To determine contents of dendritic cells, SAC were detached by EDTA and analysed by cytofluorometry after staining with anti-mouse CD11c (N418) and FITC-conjugated goat anti-hamster IgG. They were estimated to be dendritic cells at 30–45%.

‡ Macrophages were prepared from the remaining adherent cells after overnight culture of SAC by the incubation with EDTA, followed by depletion of dendritic cells. 20–35% of macrophages were found to be Ia^+. Macrophages were added to the culture at 10^5/well in both (A) and (B).

§ Fresh dendritic cells were obtained from 2-hr adherent spleen cells by detaching with EDTA followed by depleting Fc receptor positive cells with EA-rosette formation. Percentage of anti-$CD11c^+$ cells was estimated to be 70–90% and was added to the culture at 10^5/well.

¶ Anti-KLH IgG antibody was determined by ELISA method. Levels of anti-KLH IgG were expressed as DTg U/200 μl. Each value represents the mean ± SD of the duplicate assays of triplicate cultures of five separate experiments. Values 1·0 or more were considered as positive.

** T/B cells (5×10^6/culture) were prepared as G10-non-adherent cells from mice primed with 5μg of KLH 8 months before culture.

cultured with normal mice-derived SAC. This was also confirmed by the experiment in which a mixture of transgenic B cells and KLH-primed normal T cells responsed to KLH in the presence of normal SAC but not transgenic SAC. These results demonstrate the defect in antigen-presenting activity of SAC

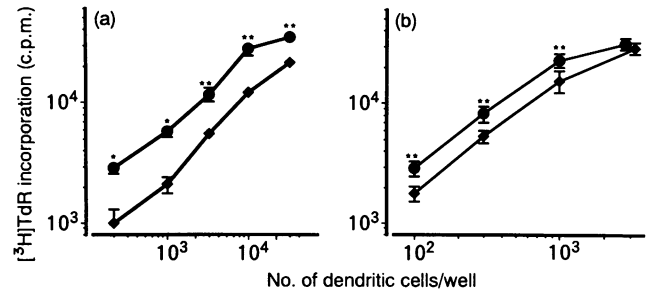

Figure 3. Inefficient stimulatory activity of HBV-transgenic dendritic cells in T-cell proliferative response. Purified T cells prepared from normal C57BL/6 mice [$NaIO_4$-induced T-cell proliferation (a)] or C3H/He mice [allogenic MLR (b)] were cultured at 3×10^5/well with graded doses of dendritic cells either from HBV-transgenic mice (◆) or normal mice (●) in 96-well culture plates. For the assay of periodate-induced T-cell proliferation (a), T cells were treated with sodium periodate prior to onset of culture as described previously.[18] [^{3}H]Thymidine was added to measure DNA synthesis at 32–46 hr (a) and 80–94 hr (b). Each symbol and bar represent the mean ± SD of the triplicate cultures of five experiments. Significant statistical differences at * $P<0·01$ and ** $P<0·05$ (Student's t-test).

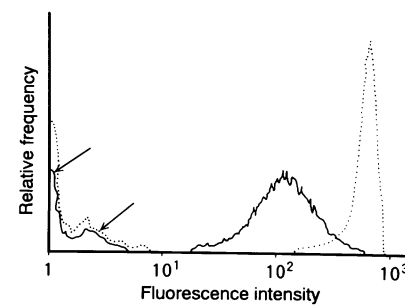

Figure 4. Decreased expression of Ia antigen on dendritic cells from HBV-transgenic mice. Spleen dendritic cells isolated from normal (· · · ·) and HBV-transgenic (——) mice were stained with anti-Ia mAb by an indirect method and Ia expression was analysed by cytofluorometer. Arrows indicate fluorescence intensity of cells stained with the second antibody alone. Each of a representative staining pattern was shown here. In most cases, more than 90% of each population was stained with hamster anti-mouse CD11c mAb (N418)[15] and FITC-conjugated goat anti-hamster Ab (not shown).

from transgenic mice in anti-KLH Ab response. We further compared the helper activities of T cells from KLH-primed normal and transgenic mice by adding them to cultures of transgenic B cells and normal SAC. Normal mice-derived T cells appeared to be slightly, but not significantly, better in helper activity than T cells from transgenic mice. In KLH-specific proliferative responses of these two cell populations in the presence of normal SAC, there were no signifcant differences between them (normal vs transgenic T cells, 26,500±5892 versus 19,115±3594 c.p.m., mean±SD of five experiments, $P>0·1$).

Critical role of dendritic cells in anti-KLH Ab response and inefficiency of transgenic dendritic cells as APC

SAC consisted of two types of cells, macrophages and dendritic cells. Dendritic cells are known to be potent in antigen

presentation. Therefore, we determined the role of dendritic cells in SAC for anti-KLH Ab response by co-culturing with lymphocytes from KLH-primed normal and transgenic mice (Table 2). In the experiments of Table 2A, depletion of normal mice-derived dendritic cells by treating with 33D1 and J11d mAb plus C either completely or almost completely abolished the anti-KLH IgG Ab responses in transgenic and normal mice, respectively, similar to that seen by depleting Ia^+ cells from normal mice-derived SAC. In addition, enriched normal mice-derived dendritic cells were able to restore the anti-KLH IgG Ab response both in normal and transgenic mice to the level found co-culturing with normal mice-derived SAC. However, enriched macrophages, although about one-quarter of them expressed Ia antigen, were incapable of inducing anti-KLH IgG Ab response by themselves. These results show that dendritic cells are a major APC for specific-Ab response.

Therefore, we constructed another set of experiments to determine whether HBV-transgenic mice-derived dendritic cells have any defect on antigen presentation (Table 2B). Like SAC from normal mice, reconstitution with normal dendritic cells resulted in the restoration of the response. In contrast, neither SAC nor dendritic cells from transgenic mice were effective as APC in the culture of transgenic lymphocytes. They, however, appeared to induce a slight response in normal lymphocytes. On the other hand, not only macrophages from normal mice but also from transgenic mice were not inhibitory to the response reconstituted with normal SAC. Normal macrophages were incapable of overcoming a defect in antigen-presenting activity of transgenic SAC.

Weak T-cell stimulatory activity of transgenic dendritic cells in sodium periodate ($NaIO_4$)-induced T-cell proliferation and allogenic MLR

Stimulatory capacities of transgenic and normal dendritic cells were compared in $NaIO_4$-stimulated non-specific (Fig. 3a) and alloantigen-induced specific T-cell proliferation (Fig. 3b). In both assays, transgenic dendritic cells were less potent in inducing responses than normal dendritic cells. Therefore, it can be concluded that transgenic dendritic cells have defect(s) in T-cell activation.

Reduced Ia antigen expression on transgenic dendritic cells

In the course of the experiments, we observed no significant difference in the proportion of Ia^+ cells between normal and transgenic SAC by monitoring with fluorescence microscopy. Therefore, we re-examined Ia antigen expression on dendritic cells by cytofluorometric analysis. One of the representative cases in each population is shown in Fig. 4 and clearly demonstrates a decreased Ia antigen expression on transgenic dendritic cells in comparison with that on normal dendritic cells. The mean fluorescence intensities with anti-Ia staining were $125{\cdot}5 \pm 22{\cdot}0$ in transgenic dendritic cells and $544{\cdot}3 \pm 89{\cdot}4$ in normal dendritic cells in four separate experiments ($P < 0{\cdot}01$, Student's *t*-test).

DISCUSSION

During progression of hepatitis or in asymptomatic HBV-carrier state, HBV genomes or their expression products have been implicated in the abnormality in the immune response.[1-5,8,9] As a result of recent progress in gene technology, transgenic mice have been constructed and used for the study of HBV-related pathogenesis.[7,21] However, these studies have mainly focused on the responsiveness of host to HBV-related antigens and there is almost no report regarding immune responsiveness to HBV-unrelated antigens in transgenic mice as well as in human HBV carriers. Transgenic mice employed here represent a suitable model for analyses of immune responsiveness to various antigens in asymptomatic HBV carriers, since they produce HBs and HBe antigens in the serum and mRNA of various sizes were detected in liver, kidney and testes without any sign of liver injury.[6]

Results in the represent study demonstrated that transgenic mice, that were completley unresponsive to HBsAg, responded poorly to KLH in a dose-dependent manner *in vivo* to produce anti-KLH Ab and their responses were significantly lower compared with the normal control mice (Fig. 1). Low responsiveness of KLH-primed spleen cells from transgenic mice was more clearly shown in *in vitro* culture (Fig. 2). This can be explained in that a small number of KLH-specific T cells were primed in transgenic mice, since they required more amounts of KLH to produce anti-KLH Ab than normal mice (Fig. 1b). The fact that T cells from KLH-primed normal mice induced comparatively higher anti-KLH Ab response than those from transgenic mice also supports this idea (Table 1).

Other investigators have demonstrated that HBV suppresses erythropoiesis and inhibits haemopoietic cell line growth.[13,14] However, we observed no decrease in numbers of bulk spleen cells, spleen lymphocytes including T and B cells, and SAC in transgenic mice compared with normal mice. No significant abnormalities were found in the structure and cellularity of lymphoid organs such as spleen, lymph node, and thymus (not shown).

Impaired polyclonal IgG synthesis has been documented in chronic HBV carriers *in vitro*,[5] and HBV-related immunosuppressive serum factor(s) have been reported as an anti-activator for normal lymphocytes.[8] Of the transgenic offspring, those that were negative for HBs and HBe antigens in the serum gave significantly higher anti-KLH Ab response than those positive for HBs and HBe antigens (not shown). Thus it is possible that an inhibitory mechanism may underlie the impairment of T-cell as well as B-cell function *in vivo*. This possibility seems to account for the apparent tendency towards low responsiveness of transgenic lymphocytes to Con A and LPS; however, the responses in transgenic mice were not significantly different from those from the normal mice. In addition, when transgenic mice were immunized with high doses of KLH in CFA, the levels of anti-KLH Ab were comparable between transgenic and normal mice (not shown). Therefore, our preferred inference is that lymphocytes are not the major responsible cell population for low anti-KLH Ab response in transgenic mice, although the possiblity of the reduction of lymphocyte function cannot be completely excluded.

Unresponsiveness of transgenic spleen cells to KLH was restored *in vitro* by co-culturing with normal spleen cells (Fig. 2) or by addition of APC from normal mice (Table 1). Furthermore, both negative and positive selection experiments using dendritic cells in Table 2 show the critical role of dendritic cells in antigen presentation as has been reported previously[22,23] and that there is impairment of antigen-presenting function of transgenic dendritic cells. The most important question is why

dendritic cells from transgenic mice were incapable of acting as efficient APC.

We observed that both transgenic and normal dendritic cells were able to endocytose comparable amounts of rhodamine-labelled ovalbumin, indicating their inefficiency in antigen presentation was not due to a decreased endocytic activity.[24] Cytofluorometric analyses demonstrated that transgenic dendritic cells expressed about four- to fivefold less amounts of Ia antigen in fluroescence intensity than normal dendritic cells (Fig. 4). Decreased Ia expression on transgenic dendritic cells correlated well with their inefficient activity for T-cell proliferation (Fig. 3). This may be one of the mechanisms leading transgenic mice to be low-, but not non-responsive to KLH, a HBV-unrelated antigen.

Our results are incompatible with those that have been reported by Takashima *et al.*[7] They mainly used hepatitis B core antigen, a HBV-related antigen, whereas we used KLH, a HBV-unrelated antigen. The dose of the antigens and the experimental design were also different in these two experiments. They found no functional defect in antigen-presenting activity of a large number of bulk spleen cells from the transgenic mice derived from the same colony by estimating hepatitis B core antigen-specific proliferative response of specific T-cell lines. This is probably attributable to differences in sensitivity of assay systems between specific T-cell lines and *in vivo*-primed T cells. Another critical difference is the source of APC. The vast majority of APC in bulk spleen cell suspensions are B cells, especially activated B cells, which are now known to present antigens fairly well to specific T-cell clones and hybridomas.[25]

We so far do not know what caused the reduction of Ia antigen expression on transgenic dendritic cells. The possibility is unlikely that the transgenic dendritic cells did not mature to activate T cells like fresh Langerhans' cells,[26] since the addition of granulocyte-machrophage colony-stimulatory factor (GM-CSF), which is known to induce maturation of dendritic cells,[27] into the culture of transgenic SAC resulted in neither improvement of T-cell stimulating activity in AMLR, nor increase in Ia antigen expression (not shown). Adenovirus has been reported to inhibit antigen presentation by interfering with transport of major histocompatibility (MHC) class I molecule.[28] Therefore there may be other mechanism(s) interfering with synthesis or transport of Ia antigen in transgenic mice due to integration and/or replication of HBV. We did not detect HBV and its products in the culture supernatant of SAC and dendritic cells. However, virus genome is integrated into mouse chromosome and there is replication of virus and expression of proteins and mRNA in sera and tissues in these mice[6] and these may have effects on the immunostimulatory capacity of dendritic cells. These points remain to be eluciated in the future.

The present investigation discloses a situation in which a virus, once integrated into the chromosome and able to replicate affected immune responsiveness of the host by interfering with the function of APC and infection with HBV, may account for decreased activity of cells participating in host defence mechanism.

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